BIOSYNTHESIS OF ASTROMICIN† AND RELATED ANTIBIOTICS

I. BIOSYNTHETIC STUDIES BY BIOCONVERSION EXPERIMENTS

SEIGA ITOH, YOSHIHIRO ODAKURA, HIROSHI KASE, SEIJI SATOH, KEIICHI TAKAHASHI, TAKAO IIDA††, KUNIKATSU SHIRAHATA and KIYOSHI NAKAYAMA

Tokyo Research Laboratories, Kyowa Hakko Kogyo Co., Ltd.,
3-6-6 Asahimachi, Machidashi, Tokyo 194, Japan

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Biosynthesis of astromicin, a unique pseudodisaccharide aminoglycoside antibiotic containing 1,4-diaminocyclitol component, was investigated by isolating a variety of possible precursor compounds from mutants of *Micromonospora olivasterospora* in which biosynthetic pathways for astromicin were blocked. Washed mycelia of *M. olivasterospora* mutants converted these compounds to astromicin, which was detected by thin-layer chromatography. Since astromicin possesses one glycyl and three methyl groups, [^{14}C]glycine and [^{14}C]methionine should be incorporated into precursors to form astromicin. To confirm the biosynthetic pathway, formation of labeled astromicin from the precursors was examined using [1-^{14}C]-glycine or [*methyl*-^{14}C]methionine. From above results, we propose the biosynthetic pathway for astromicin as shown in Fig. 2.

Astromicin (ASTM=fortimicin A) is an aminoglycoside antibiotic produced by *Micromonospora olivasterospora*, which was found by NARA and his co-workers.[1] Biosynthetic pathways for the biosynthesis of aminocyclitol antibiotics, gentamicin,[2,3] sagamicin,[4~6] neomycin,[7,8] kanamycin[9,10] and streptomycin[11,12] have been studied extensively. However, all of these aminoglycosides are pseudotrisaccharides or pseudotetrasaccharides whereas astromicin is a unique pseudodisaccharide containing the 1,4-diaminocyclitol, fortamine. Elucidation of its biosynthetic pathway contributes important additional information and also provides a way to produce new astromicin-group antibiotics by a mutasynthetic approach.[13~16] In this report, we propose a possible biosynthetic pathway for astromicin and related antibiotics.

Materials and Methods

Microorganisms Used

M. olivasterospora KY11518 was employed as the astromicin producer and KY11558 was used as the mutant strain in which the biosynthetic pathway for astromicin was blocked.

Fermentation Media and Cultivation Condition

The composition of seed medium was: Stabilose K (soluble starch) 2.5%, glucose 0.5%, peptone 1%, yeast extract 0.1%, K_2HPO_4 0.05%, $MgSO_4 \cdot 7H_2O$ 0.05% and $CaCO_3$ 0.1% in deionized water. The pH was 7.2 before autoclaving. The composition of the fermentation medium was: Stabilose K 5%, soy bean meal 3%, Ebios (dried yeast) 1.5%, $MgSO_4 \cdot 7H_2O$ 0.05%, KCl 0.03%, K_2HPO_4 0.05%, mannitol 0.05%, $CoCl_2 \cdot 6H_2O$ 500 μg/liter, $NiCl_2 \cdot 6H_2O$ 50 g/liter, Ca-Mg_2-phytate 0.02%, Arg·HCl 0.1%, His·HCl 0.05% and $CaCO_3$ 0.1% in deionized water. The pH was 7.2 before autoclaving. All media were sterilized by autoclaving at 120°C for 40 minutes. First seed culture were developed

† Astromicin was initially designated as fortimicin A.

†† Technical Research Laboratories, Hofu Plant, Kyowa Hakko Kogyo Co., Ltd., 1-1 Kyowamachi, Hofu-shi, Japan.

in a large test tube (2.5×19 cm) containing 10 ml of the seed medium by shaking at 30°C for 3 days. A portion (3 ml) of the seed culture was transferred into a large test tube containing 30 ml of the seed medium and shaken at 30°C for 3 days. A 3-ml portion of the second seed culture was transferred to 300-ml Erlenmeyer flasks containing 30 ml of the fermentation medium. Fermentation was carried out at 30°C for 4~5 days with shaking at 220 rpm.

Bioconversion of a Precursor to Astromicin by the Washed Mycelia of *M. olivasterospora*

Wild and mutant strains of *M. olivasterospora* were cultivated in the fermentation medium for 3 days. The mycelia were collected by centrifugation and washed twice with 0.1 M Tris-HCl buffer (pH 7.3). The washed mycelia were suspended in Tris-HCl buffer (pH 7.3) containing 0.2% glucose and used for the bioconversion experiments. The reaction mixture containing 60 mg (dry weight) cells and 800 μg substrate in 2 ml of Tris-HCl buffer (pH 7.3) was incubated with shaking at 30°C for 20~24 hours. The bioconversion products in the reaction mixture were detected by thin-layer chromatography (TLC) on a Merck Silica Gel 60 plate using the lower phase of chloroform - methanol - conc ammonium hydroxide (1:1:1) as a solvent system. Astromicin and related compounds were visualized by the Rydon-Smith reaction[17] and measured fluorometrically by scanning with a Shimadzu CS-910 double-beam densitometer. For tracer experiments, 200 μl of the washed mycelial suspension, 20 μl of solution containing astromicin precursor (4 mg/ml) and 0.5~1 μCi of [1-^{14}C]glycine or [*methyl*-^{14}C]methionine was mixed in a small test tube and shaken 18~20 hours at 30°C. The suspension was centrifuged after adjusting to pH 2.0 with oxalic acid and the supernatant was submitted to silica gel thin-layer chromatography. Formation of astromicin was detected by measuring the incorporation of labeled substance into the spot corresponding to astromicin.

Isolation of Fermentation Products

Astromicin-related compounds were isolated from the mutants blocked in different steps of astromicin biosynthesis. FU-10, one of the possible precursor compounds, was isolated as follows: 15 liters of fermentation broth of *M. olivasterospora* mutant strain was adjusted to pH 2.0 with 6 N HCl and then adjusted to pH 7.0 with sodium hydroxide. Filter aid (Radiolite 600, Showa Kako Co., Ltd., Japan) was added to the broth and the mixture was filtered. The filtrate was passed through the cation-exchange resin Amberlite IRC-50 (NH_4^+). After washing the resin column with deionized water, fermentation products were eluted with 1 N ammonium hydroxide. Fractions containing astromicin precursor were collected and concentrated *in vacuo* to 1/3~1/5 of the original volume. The concentrate was passed through a column of the cation-exchange resin Amberlite CG-50 (NH_4^+) and washed with deionized water. Astromicin-related antibiotics were eluted with a linear gradient of 0.05 to 0.5 N ammonium hydroxide. Fractions were monitored by thin-layer chromatography and those containing the astromicin-related compound (FU-10) were concentrated, lyophilized and purified further by silicic acid column chromatography, if necessary. Isolation and characterization of other astromicin-related substances such as FTM-KR, FTM-KH, FTM-AP, FTM-KK_1, FTM-KL_1, and FTM-AO was carried out by a similar procedure. Structural analysis of these substances will be published elsewhere.

Results

Bioconversion of Various Precursors to Astromicin

To establish a biosynthetic pathway for astromicin, we first examined the production of astromicin from various compounds which were isolated from the wild or mutant strains of *M. olivasterospora*. By use of washed mycelia prepared from 3-day cultures of mutant strain KY11558, several compounds such as FU-10, FTM-AO, FTM-KK_1, FTM-AP, FTM-KH, FTM-KR and FTM-B were converted to astromicin (Table 1). Formation of ASTM was detected by bioautography and silica gel thin-layer chromatography and one of the thin-layer chromatograms is shown in Fig. 1. FTM-KK_1, FTM-KH or FTM-KR was transformed to FTM-B, when the resting cells were prepared from KY11582, which

Table 1. Biotransformation of precursors to astromicin with the resting cells of a blocked mutant KY11558.

The reaction mixture contained 200 μg of substrate and 15 mg of washed mycelia in 550 μl of 0.1 M Tris-HCl buffer (pH 7.3) containing 0.2% glucose. The reaction was carried out at 30°C for 20 hours with shaking.

Substrate	Astromicin produced (μg/ml)
None	Trace
FU-10	15
FTM-AO	8
FTM-KK_1	9
FTM-AP	13.5
FTM-KH	11.5
FTM-KR	21.5
FTM-B	19

Table 2. Biotransformation of biosynthetic precursors to astromicin detected by [^{14}C]-amino acid incorporation.

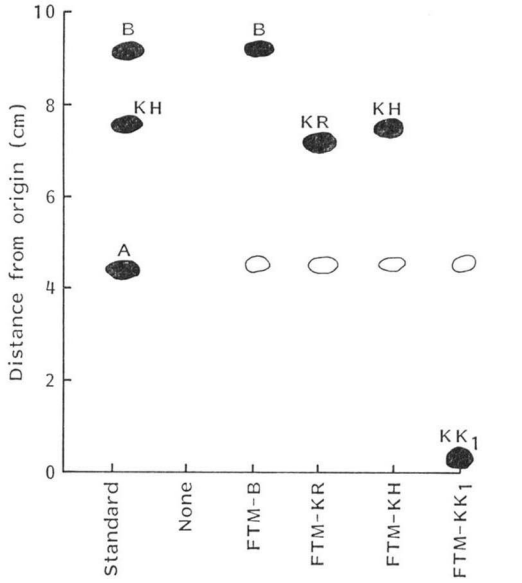
Reaction mixture: 6 mg of washed mycelia of *M. olivasterospora* KY11581, 80 μg of substrate and 0.5 μCi of [1-^{14}C]glycine (58 mCi/mmol) or 0.5 μCi of L-[*methyl*-^{14}C]methionine (55 mCi/mmol) in 200 μl of 0.1 M Tris-HCl buffer (pH 7.3) containing 0.2% glucose. The reaction was carried out 30°C for 21 hours with shaking. The amount of [^{14}C]astromicin was determined by measuring the radioactivity recovered from the spot corresponding to astromicin on thin-layer chromatography.

Expt No.	Substrate	[^{14}C]-Amino acid	[^{14}C]-Astromicin produced (cpm)
1	None	[^{14}C]Glycine	22
	FU-10		697
	FTM-AO		768
	FTM-KK_1		547
	FTM-AP		840
	FTM-KH		834
	FTM-KR		1,107
	FTM-B		1,235
2	None	[^{14}C]Methio-	144
	FU-10	nine	1,553
	FTM-AO		2,101
	FTM-KK_1		1,531

Fig. 1. Silica gel thin-layer chromatogram of astromicin produced from precursors in washed mycelia of *M. olivasterospora* KY11581.

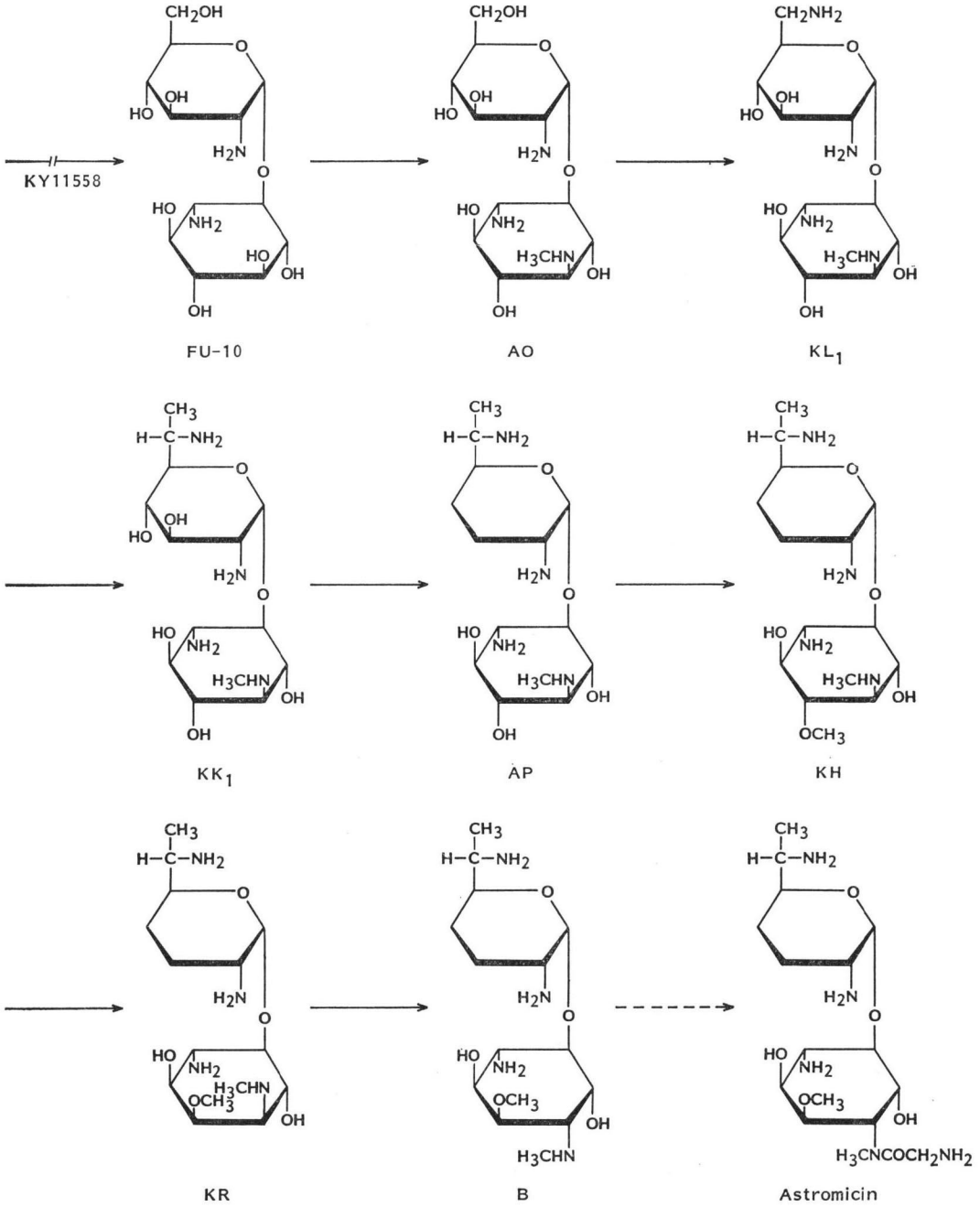
Solvent system: lower phase of $CHCl_3$ - CH_3OH - 17% NH_4OH (1 : 1 : 1).

Detection: Rydon-Smith reaction.[17]



lacks the ability to convert FTM-B to astromicin (unpublished results). Astromicin possesses an *N*-glycyl group which is not present in FTM-B. Assuming that glycine is the donor of glycyl group of astromicin, one molecule of glycine should be incorporated into FTM-B to form astromicin. Therefore, incorporation of [^{14}C]-glycine into astromicin was examined by incubating substrates with washed mycelia of *M. olivasterospora* KY11558. Table 2 shows that radiolabeled astromicin was produced when [^{14}C]-glycine was incubated with FU-10, FTM-AO, FTM-KK_1, FTM-AP, FTM-KH, FTM-KR and FTM-B, respectively. Bioconversion of FU-10, FTM-AO and FTM-KK_1 to astromicin was also confirmed using [^{14}C]methionine, assuming that the radioactivity from L-[*methyl*-^{14}C]methionine would be incorporated into *C*-methyl and *N*-methyl groups of astromicin. Biosynthesis of astromicin from the other precursors, such as FTM-KL_1, was confirmed in independent experiments (data are not

Fig. 2. Proposed biosynthetic pathway for astromicin.



shown). Based on the above findings as well as other experiments described in the accompanying paper, the possible biosynthetic pathway for astromicin shown in Fig. 2 is proposed. The formation of FU-10 from precursors and characterization of mutants blocked in astromicin biosynthesis will be described in the accompanying papers.

Discussion

Among aminoglycosides, biosynthetic pathways have been extensively studied with gentamicin, neomycin and kanamycin. It has been shown that these aminoglycosides are synthesized *via* intermediates which are formed from 2-deoxystreptamine (DOS) and an aminosugar. For example, gentamicin C_1 is synthesized *via* paromamine which is consist of DOS and glucosamine. Neomycin is also synthesized *via* neamine which contains DOS and neosamine. Taking the above pathways into consideration, we first assumed that astromicin would be synthesized *via* 4-amino-FU-10 which contains fortamine (1,4-diaminocyclitol) and glucosamine. However, a possible precursor compound FU-10 which contains 1-aminocyclitol and glucosamine was isolated in many mutants and the structure of FU-10 suggests that astromicin is synthesized *via* a unique pathway (Fig. 2), different from DOS-containing aminoglycosides. The biosynthetic pathway for astromicin proposed above is based on experiments with washed mycelia, but synthesis of astromicin from FTM-B was confirmed using a cell free system. Further investigations are underway to elucidate the biosynthetic pathway for astromicin including a detailed analysis of the conversion of FTM-B to astromicin.

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